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(54) Alcohol/aldehyde dehydrogenase.

(57) The present invention relates to a novel alcohol/aldehyde dehydrogenase (hereinafter referred to as AADH), a process for producing the same and a process for producing aldehydes, carboxylic acids and ketones, especially 2-keto-L-gulonic acid (hereinafter referred to as 2-KGA) utilizing said enzyme.

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The present invention relates to a novel alcohol/aldehyde dehydrogenase (hereinafter referred to as AADH), a process for producing the same and a process for producing aldehydes, carboxylic acids and ketones, especially 2-keto-L-gulonic acid (hereinafter referred to as 2-KGA) utilizing said enzyme.

The AADH provided by the present invention catalyzes the oxidation of alcohols and aldehydes, and is thus capable of producing the corresponding aldehydes and ketones from alcohols, and carboxylic acids from aldehydes. More particularly, the AADH provided by the present invention catalyzes the oxidation of L-sorbose to 2-KGA via L-sorbosone. 2-KGA is an important intermediate for the production of vitamin C.

It is known that there are enzymes which catalyze the oxidation of alcohols and aldehydes to aldehydes and carboxylic acids, respectively, and have pyrroloquinoline quinone (hereinafter referred to as PQQ) as prosthetic group.

Methanol dehydrogenases, which are members of alcohol dehydrogenases, catalyze in common not only the oxidation of methanol to formaldehyde, but also that of formaldehyde to formic acid and formate respectively (Advances in Microbial Physiology, 27, 113-209, 1986). These enzymes oxidize a wide range of primary alcohols such as methanol and ethanol and some aldehydes using ammonia or methylamine as activator, but most of them cannot oxidize secondary alcohols. The methanol dehydrogenases derived from Methylobacterium organophilum, Pseudomonas C, Diprococcus PAR, and Rhodopseudomonas acidophile are examples for dehydrogenases which can catalyze the oxidation of secondary alcohols as well. In contradistinction thereto, the AADH provided by the present invention oxidizes a wide range of primary and secondary alcohols, and is clearly distinct from the previously mentioned methanol dehydrogenases in the following aspects.

AADH hardly oxidizes methanol, while ethanol is a good substrate. It requires neither ammonia nor methylamine as activator. The isoelectric point of the novel AADH is about 4.4, while that of most of methanol dehydrogenases is higher than 7.0.

As other examples of alcohol dehydrogenases having PQQ as prosthetic group, quinoprotein alcohol dehydrogenase from Pseudomonas aeruginosa (Biochem. J., 223, 921-924, 1984) and quinohaemprotein alcohol dehydrogenase from Pseudomonas testosteroni (Biochem., J., 234, 611-615, 1986) are known. The former enzyme is a monomer whose molecular weight is 101,000 and requires ammonium salts or amines as activators, while AADH provided by the present invention consists of two subunits and does not require any activator. The latter enzyme is a monomer whose molecular weight is about 67,000 containing one heam c group and one PQQ as prosthetic group in its molecule, while the AADH provided by the present invention consists of two subunits and does not contain a heam c group.

As described above, there have been no reports up to now concerning an AADH as provided by the present invention. It has been found that the purified enzyme isolated from a cytosol fraction of cells of specific microorganisms catalyzes the oxidation of alcohols and aldehydes, and is capable of producing aldehydes and ketones from alcohols, and carboxylic acids from aldehydes. More particularly, the enzyme in question catalyzes the oxidation of L-sorbose to 2-KGA via L-sorbosone. The present invention has been accomplished on the basis of this finding.

The physico-chemical properties of the purified sample of AADH prepared by Examples mentioned hereinafter are as follows:

1) Enzyme activity

The AADH of the present invention catalyzes the oxidation of alcohols and aldehydes, and is capable of producing aldehydes and ketones from alcohols, and carboxylic acids from aldehydes in the presence of an electron acceptor.

The enzyme does not utilize oxygen as an electron acceptor. However, any conventional compound which has the ability to act as an electron acceptor can be utilized together with the enzyme of this invention. As an electron acceptor, 2,6-dichlorophenolindophenol, (hereinafter referred to as DCIP), phenazine methosulphate (hereinafter referred to as PMS), Wurster's blue, ferricyanide, coenzyme Q or cytochrome c can be used.

The enzyme assay was performed at 25°C by measuring the decrease of absorbance at 600 nm of DCIP with a spectrophotometer (UVIKON 810, Kontron K.K.). One unit of the enzyme activity was defined as the amount of the enzyme which catalyzed the reduction of 1 μ mole of DCIP per minute. The extinction coefficient of DCIP at pH 8.0 was taken as 15 $mMol^{-1}$. The standard reaction mixture (1.0 ml) contained 0.1 mMol DCIP, 1 mMol PMS, 125 mMol L-sorbose, 50 mMol Tris-malate-NaOH buffer (pH 8.0), and 3-8 μl of the enzyme solution. A reference cuvette contained all the above components except the substrate.

2) Substrate specificity

The substrate specificity of AADH was determined using the same enzyme assay method as described above under 1) except that various further substrates were used instead of L-sorbose. The results of the measurement are shown in Table 1. A variety of compounds such as primary alcohols, secondary alcohols, aldehydes and high molecular weight alcohols such as polyethylene glycols or polyvinyl alcohols can be the substrates for AADH.

3) Optimum pH

The correlation between the reaction rate of AADH and pH was determined in Tris-malate-NaOH buffer (pH 6.0 to 8.5) and in Tris-HCl buffer (pH 9.0) using a variety of substrates as shown in Table 2. Regardless of the kind of the substrates, AADH showed the highest activity at a pH range between 7.0 and 9.0.

4) pH stability

Purified AADH was kept standing in buffers of various pH-values for a certain period at 4 • C as shown in Table 3. The residual activity was assayed under the standard assay condition as described above under 1) using L-sorbose and L-sorbosone as the substrates. The results of the measurements are shown in Table 3. The purified enzyme was relatively stable in alkaline pH's and became unstable with increased acidity.

5) Heat stability

Purified AADH was treated for 10 minutes at various temperatures in 25 mMol Tris-HCl buffer (pH 8.0) containing 0.1 Mol NaCl and 5% sucrose, and then cooled immediately in ice water. The residual activity was measured under the standard assay conditions as described under 1) using a variety of substrates. The results are shown in Table 4. AADH is rather stable at and below 30 • C, while unstable above 40 • C.

6) Optimum Temperature

The enzymatic activities of AADH were measured at temperatures from 10 • C to 50 • C in the reaction system as described under 1) above using a variety of substrates. The results are shown in Table 5. This enzyme showed its optimum et temperatures between 20 • C and 40 • C.

7) Molecular Weight

The molecular weight of purified AADH was determined by gel filtration column chromatography. The sample was applied on a resin for the purification of proteins, e.g. Sephacryl S-300HR (Pharmacia) column equilibrated with 25 mMol Tris-HCl buffer (pH 8.0) containing 0.1 Mol NaCl and 5% sucrose. As molecular weight standards, thyroglobulin (670,000 dalton), ferritin (450,000 dalton), catalase (240,000 dalton), aldolase (158,000 dalton), gamma globulin (158,000 dalton), bovine serum albumin (66,200 dalton), ovalbumin (45,000 dalton), chymotrypsinogen A (25,000 dalton), myoglobin (17,000 dalton), cytochome c (12,500 dalton) and vitamin B_{12} (1,359 dalton) were used. As a result, the molecular weight of AADH was determined to be 135,000 ± 5,000 dalton. Next, purified AADH was treated by sodium dodecyl sulfate (SDS) in the presence of beta-mercaptoethanol and analyzed for its molecular structure by SDS-polyacrylamide gel electrophoresis. As molecular weight standards, phosphorylase B (92,500 dalton), bovine serum albumin (66,200 dalton), ovalbumin (45,000 dalton), carbonic anhydrase (31,000 dalton), soybean trypsin inhibitor (21,500 dalton) and lysozyme (14,400 dalton), were used. It was shown that the enzyme consists of two subunits. One (α-subunit) has a molecular weight of 64,500 ± 2,000 dalton and the other (β-subunit) 62,500 ± 2,000 dalton.

8) Measurement of the Km (Michaelis constant) values

In the procedure described under 1), the velocities of oxidizing reactions with varying concentrations of several substrates were measured to determine the apparent Michaelis constant (Km). The mixture of DCIP and PMS was used as electron acceptors. The Km values for L-sorbose and 1-propanol were calculated to be 230 mMol and 2 mMol, respectively.

9) Effect of metal ions

Using the assay procedure described under 1), the effect of various metal ions on the enzyme activity was examined. The results of the measurement are shown in Table 6. Among the ions tested, only Mg^{2+} and Ca^{2+} did not affect the AADH activity. The others affected the enzyme activity strongly or moderately. Cu^{2+}, Mn^{2+} and Fe^{3+} are strong inhibitors for the enzyme.

10) Effect of inhibitors

Using the assay procedure described under 1) above, the effect of inhibitors on the enzyme activity was examined. The results are shown in Table 7. Ethylenediamine tetraacetic acid (EDTA) and ethylene glycol bis(beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) strongly inhibited the enzyme activity.

11) Prosthetic group

The absorption spectrum of purified AADH showed an absorption maximum at 280 nm followed by a shoulder at 290 nm as shown in Fig. 1. The second peak was detected at 340 nm with a wide shoulder at 380-420 nm. This absorption spectrum strongly suggested that AADH has PQQ as a prosthetic group.

Purified AADH (4.5 mg) in 100 mMol NaH_2PO_4-HCl (pH about 1.0) was added to an equal volume of methanol and mixed. The sample was then centrifuged at 15,000 rpm for 10 minutes to remove the precipitate. The resulting extract was used for the analysis of the prosthetic group. The absorption spectrum of the extract was completely identical with an authentic sample of PQQ (Mitsubishi Gas Chemical Co.). Furthermore, by high pressure liquid chromatography analysis, using a reverse phase column (TSK-ODS 80 TM, Toyo Soda CO.), the extract from AADH showed the same retention time as that of authentic PQQ.

12) Isoelectric point

The isoelectric point (pI) of AADH was determined. Polyacrylamide gel (4%) containing 8.5 Mol urea, 2% (w/v) a non-ionic detergent, e.g. Nonidet P-40 and 2.4% (w/v) of an Ampholite, a buffer component for the pH-gradient, namely Pharmalyte, pH 2.5-5.0 (Pharmacia), was used for isoelectric focussing. The electrode solutions were 0.01 Mol iminodiacetate for the anode and 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer for the cathode. The isoelectric point of the sample was estimated in comparison with a low pH calibration kit, pH 2.5-5.0, purchased from Pharmacia. As a result, AADH showed a cluster containing a few bands having pI points of about 4.4.

13) Purification method

The purification of AADH may, in principle, be effected by any combination of known purification methods such as

fractionation with precipitants, e.g. ammonium sulfate, polyethylene glycol, etc.
ion exchange chromatography,
adsorption chromatography,
gel-filtration chromatography,
gel electrophoresis,
salting out and dialysis.

The AADH provided by the present invention can be prepared by cultivating an appropriate microorganism, disrupting the cells and isolating and purifying it from cell free extract of disrupted cells, preferably from the cytosol fraction of the microorganism.

The microorganisms used in the present invention includes all strains belonging to the genus Gluconobacter which are capable of producing AADH hereinbefore. Functional equivalents, subcultures, mutants and variants of said microorganism can be also used in the present invention.

A preferred strain is Gluconobacter oxydans. A specific and preferred Gluconobacter oxydans strain has been deposited at the Deutsche Sammlung von Mikroorganismen in Göttingen (Germany) under DSM No. 4025 on March 17, 1987.

Moreover, a subculture of the strain has also been deposited in the Agency of Industrial Science and Technology, Fermentation Research Institute, Japan, under the stipulations of the Budapest Treaty under the deposit No.:

Gluconobacter oxydans DSM No. 4025 FERM BP-3812 (date of deposit: March 30, 1992).

Furthermore, European Patent Publication No. 0278 447 (4226/081k) discloses the characteristics of this strain.

The microorganisms may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at pH's is between about 4.0 and 9.0, preferably between about 6.0 and 8.0. While the cultivation period varies depending upon pH, temperature and nutrient medium used, usually 2 to 5 days will bring about favorable results. A preferred temperature range for carrying out the cultivation is from about 13° to 36°C, preferably from about 18° to 33°C.

It is usually required that the culture medium contains such nutrients as assimilable carbon sources, digestable nitrogen sources and inorganic substances, vitamins, trace elements and other growth promoting factors. As assimilable carbon sources, L-sorbose, glycerol, D-glucose, D-mannitol, D-fructose, D-arabitol and the like can be used.

Various organic or inorganic substances may also be used as nitrogen sources, such as yeast extract, meat extract, peptone, casein, corn steep liquor, urea, amino acids, nitrates, ammonium salts and the like. As inorganic substances, magnesium sulfate, potassium phosphate, ferrous and ferric chlorides, calcium carbonate and the like may be used.

In the following, an embodiment for the isolation and purification of AADH from microorganisms after the cultivation is briefly described.

(1) Cells are harvested from the fermentation broth by centrifugation or filtration.

(2) The cells are suspended in the buffer solution and disrupted by means of a homogenizer, sonicator or treatment with lysozyme and the like to give a disrupted solution of cells.

(3) AADH is isolated and purified from a cell free extract of disrupted cells, preferably from the cytosol fraction of the microorganisms.

The AADH provided by the present invention is useful as a catalyst for the production of aldehydes, carboxylic acids and ketones from alcohols and aldehydes, especially for the production of 2-KGA from L-sorbose via sorbosone.

The reaction should be conducted at pH values of from about 6.0 to about 9.0 in the presence of an electron acceptors, for example, DCIP, PMS, Wurster's blue, ferricyanide, coenzyme Q, cytochrome c and the like in a solvent such as Tris-HCl buffer, phosphate buffer and the like.

A preferred temperature range for carrying out the reaction is from about 10°C to about 50°C. When the pH and the temperature are set at about 7.0-8.0 and 20° - 40°C, respectively, the reaction usually brings about the most preferable results.

The concentration of the substrate in a solvent can vary depending on other reaction conditions but, in general, is desirable to be about 10-100 g/l, most preferably from about 30-40 g/l.

In the reaction, AADH may also be used in an immobilized state with an appropriate carrier. Any means of immobilizing enzymes generally known to the art may be used. For instance, the enzyme may be bound directly to a membrane, granules or the like of a resin having functional group(s), or it may be bound through bridging compounds having functional group(s), for example, glutaraldehyde, to the resin.

In addition to the above, the cultures cells are also useful for the production of aldehydes, ketones and carboxylic acids from alcohols and aldehydes, especially for the production of 2-KGA from L-sorbose.

The following examples further illustrate the present invention.

Example 1

Preparation of AADH

All the operations were performed at 4°C unless otherwise described.

(1) Cultivation of Gluconobacter oxydans DSM No. 4025 (FERM BP-3812)

(α) Preparation of the medium

A seed culture medium containing L-sorbose 8% (w/v) (separately sterilized), glycerol 0.05%, $MgSO_4 \cdot 7H_2O$ 0.25%, corn steep liquor 1.75%, baker's yeast 5.0%, $CaCO_3$ 0.5% and urea 0.5% (separately sterilized) (pH 7.0 before sterilization) was put into a test tube (5 ml each) and sterilized at 120°C for 20 minutes.

(β) Inoculation, Incubation

Into this seed culture medium, one loopful of the cells of Gluconobacter oxydans DSM No. 4025 (FERM BP-3812) grown on a slant culture medium containing D-mannitol 5.0% in water, $MgSO_4 \cdot 7H_2O$ 0.25%, corn steep liquor 1.75%, baker's yeast 0.25%, $CaCO_3$ 0.5%, urea 0.5% (separately sterilized) and agar 2.0%, (pH 7.0 before sterilization) at 27 • C for four days was inoculated and incubated at 30 • C for 24 hours. The resulting seed culture (5 ml) was inoculated into 100 ml of the same seed culture medium as described above in a 500 ml-Erlenmeyer flask and incubated at 30 • C for 24 hours. Further, the resulting seed culture (5 ml) was inoculated into 100 ml of the same seed culture medium as described above in a 500 ml-Erlenmeyer flask and incubated at 30 • C for 24 hours. 750 ml of the seed culture thus prepared were used for the inoculation of 15 l of a main medium in a 30 l jar fermentor. The medium contained L-sorbose 10.0% (sterilized separately), glycerol 0.05%, urea 1.6% (sterilized separately), $MgSO_4 \cdot 7H_2O$ 0.25%, baker's yeast 5.0%, $CaCO_3$ 1.5% and corn steep liquor 3.0%. The fermentation was carried out at 30 • C, 500 rpm for the agitation and 7.5 l/minute for the aeration. Alter 40 hours fermentation, the culture was harvested by centrifugation (10,000 g, 15 minutes). The cell cake was suspended in 1 l of 25 mMol Tris-HCl, pH 7.5, containing 0.9% NaCl, 5 mMol $MgCl_2$ and 1 mMol phenylmethylsulfanyl fluoride (PMSF). The suspension was centrifuged at 500 g for 5 minutes to precipitate down $CaCO_3$ and other precipitatable medium ingredients. Then, the cells were collected by centrifugation at 10,000 g for 15 minutes. The operation as mentioned above was repeated again. As a result, 125 g (wet weight) of the cells of Gluconobacter oxydans DSM No. 4025 (FERM BP-3812) were obtained. The washed cells were frozen at -20 • C for one week before the next purification step.

(2) Preparation of the cytosol fraction

The cells of Gluconobacter oxydans DSM No. 4025 (FERM BP-3812) (125 g) from the above step (1) were suspended in 100 ml of 25 mMol Tris-HCl buffer, pH 8.0, containing 0.5 mMol PMSF and subjected twice to a (French press) cell disrupter to break the cells (1,500 kg/cm^2). Into this homogenized suspension, 2 ml of 1 mg/ml of the DNA splitting DNase I (Sigma) and 1 ml of 0.5 Mol $MgCl_2$ were added, the mixture kept standing for 15 minutes and centrifuged at 6,000 g for 15 minutes to remove the cell debris. The cell free extract (210 ml) thus obtained was centrifuged at 100,000 g for 60 minutes. The resulting supernatant was collected as the cytosol fraction (200 ml).

(3) PEG (MW 6000) treatment (precipitation of the DNA)

The cytosol fraction (200 ml) from step (2) was dialyzed overnight against 2 liters of 25 mMol Tris-HCl buffer, pH 8.0, containing 0.25 mMol PMSF; then 40 g of PEG 6000 (Nakarai Chemicals Ltd.) and 5 ml of 2N KCl, were added and the mixture stirred for 30 minutes and centrifuged at 14,000 g for 20 minutes. The supernatant was filled up to 400 ml with the same buffer.

(4) DEAE Toyopearl 650M (weak ion-exchange) column chromatography [first step]

The supernantant (400 ml) obtained from the above step (3) was applied to a diethylaminoethyl (DEAE) Toyopearl 650M column (2.5 cm in diameter and 35 cm in length), which had been equilibrated with 25 mMol Tris-HCl buffer, pH 8.0, containing 0.25 mMol PMSF and 5% sucrose. Alter the column was washed with 600 ml of the same buffer, the enzyme was eluted by a linear gradient of NaCl from 0 to 0.5 Mol in the same buffer (2,000 ml). The active fractions were pooled (174 ml) and subjected to the next step.

(5) Q-Sepharose (strong-ion exchange) column chromatography [second step]

The active fractions in the previous step were applied to a Q-Sepharose column (2.5 cm in diameter and 35 cm in length) which had been equilibrated with 25 mMol Tris-HCl buffer, pH 8.0, containing 5% sucrose. After the column was washed with the buffer to the baseline, the elution of the enzyme was performed with a linear gradient of 0.25 to 0.5 Mol NaCl in the same buffer (2,000 ml). The fractions which contained electrophoretically homogenous AADH were combined and concentrated to 20 nil by ultra-filtration using a PM-30 (Amicon Corporation) membrane.

The summary of the purification steps of AADH is shown in Table 8.

(6) Purity of isolated AADH

For the estimation of the purity of isolated AADH, a polyacrylamide gel electrophoresis was performed. The sample was applied to 7.5% polyacrylamide gel in Tris-HCl buffer, pH 9.4, according to the procedure of Davis et al. (Ann. N.Y. Acad. Sci. 121: 404, 1969). Proteins were stained with the protein colourant Coomassie Brilliant Blue R-250. The enzyme activity in the gel was detected by coupling it under the reduction of nitro blue tetrazolium chloride (Sigma). The gel was immersed at 30 °C in the dark in a solution containing 50 mMol Tris-malate buffer, pH 8.0, 0.01 mMol PQQ, 0.1 mMol PMS, 0.4 mMol nitroblue tetrazolium chloride and 0.25 Mol L-sorbose.

AADH showed the closely spaced three bands by protein staining, and all the bands had enzyme activity. The appearance of three protein bands on the gel is due to the dissociation of the prosthetic group, PQQ, from the enzyme during electrophoresis.

(7) Identification of reaction product

A reaction mixture containing 50 µl of the purified AADH (1.5 mg protein), 0.1 ml of 10 mMol PMS, 0.5 ml of 0.4 Mol sodium phosphate buffer, pH 6.5, 0.25 ml of water and 0.1 ml of 20% solution of the various substrates was incubated at 30 °C for 15 hours with gentle shaking. The reaction product was analyzed by thin layer chromatography. Products were identified by the direct comparison with authentic samples. The result was summarized in Table 9.

Example 2

2-KGA production by purified AADH

A reaction mixture containing 0.5 ml of purified AADH (15 mg protein, and prepared according to Example 1), 1 ml of a 20% solution of L-sorbose, 1 ml of 10 mMol PMS, 5 ml of 0.4 M sodium phosphate buffer, pH 6.5 and 2.5 ml of water was incubated at 30 °C with gentle shaking. As a result, 2-KGA was formed with the rate of about 70 mg/hour.

Example 3

2-KGA production under a resting cell system

The reaction mixture (10 ml): 0.25 g of the cells of Gluconobacter oxydans DSM No. 4025 (FERM BP-3812) prepared in the same manner as described in step (1) of Example 1, 1 ml of a 20% solution of L-sorbose, 1 ml of 10 mMol PMS, 1 ml of a 3% aqueous solution of NaCl, 1 ml of 30 µMol PQQ, 0.1 g of $CaCO_3$ and water, was incubated at 30 °C with gentle shaking. As a result, 2-KGA formation was observed with the rate of about 6 mg/hour.

Table 1

Substrate Specificity of AADH		
Substrate	Concentration (mM)	Relative Activity(%)
L-sorbose	125	10.9
L-sorbosone	2	16.2
D-sorbitol	50	7.1
D-glucose	50	25.3
D-mannitol	50	22.2
D-fructose	125	6.6
DL-glycelaldehyde	25	80.2
Methanol	50	0.3
Ethanol	50	88.6
1-Propanol	50	100.0
1-Butanol	50	69.4
1-Pentanol	50	49.8
1-Hexanol	50	49.1
1-Heptanol	50	47.7
2-Propanol	50	78.2
2-Butanol	50	87.4
Propionaldehyde	25	83.3
PEG*1000	0.4 %	68.9
PEG*2000	0.4 %	60.2
PEG*4000	0.4 %	34.5
PEG*6000.	0.4 %	14.1
PVA**	0.4 %	38.1

* Polyethylene glycol (Maker: Nakarai Chemicals Ltd.)
** Polyvinyl alcohol (Maker: Wako Pure Chemical Industries, Ltd.)

Table 2

Optimum pH of AADH					
	Relative Activity (%)				
Substrate (mM) / pH	Sorbose 125	Sorbosone 2	Sorbitol 125	Glucose 50	1-Propanol 50
6.0	19.4	26.1	51.2	6.6	2.9
6.5	34.2	47.7	78.6	14.0	6.7
7.0	50.6	56.4	90.1	32.0	16.0
7.5	80.9	70.5	100.0	51.2	28.5
8.0	94.4	100.0	92.2	100.0	73.7
8.5	100.0	77.1	82.2	84.2	100.0
9.0	41.7	48.7	37.7	76.6	83.6

Table 3

pH Stability of AADH					
		Residual Activity (%)			
Storage Period		1 day		5 days	
Substrate		Sorbose	Sorbosone	Sorbose	Sorbosone
pH	Buffer				
3.0	MC	0	0	0	0
4.0	AC	0	0	0	0
5.0	AC	46.6	45.0	41.0	19.0
6.0	TM	68.4	48.0	48.9	40.0
7.0	TM	77.5	48.0	66.1	58.4
8.0	TH	84.0	69.0	69.4	53.0
9.0	TH	77.2	75.0	70.7	48.2

Buffers: MC McIlvaine buffer
AC Acetate buffer
TM Tris-Malate-NaOH buffer
TH Tris-HCl buffer

Table 4

Heat Stability of AADH					
	Residual Activity (%)				
Substrate	Sorbose	Sorbosone	Sorbitol	Glucose	1-Propanol
Temperature (°C)					
4	100	100	100	100	100
20	93.3	106.3	101.6	97.6	105.4
30	93.3	104.9	104.8	103.4	96.1
40	57.3	62.9	66.1	69.3	58.9
50	26.7	8.4	17.7	2.0	26.4
60	10.7	2.1	16.1	0	21.6
70	0	0	8.1	0	9.7

Table 5

Optimum Temperature of AADH					
	Relative Activity (%)				
Substrate	Sorbose	Sorbosone	Sorbitol	Glucose	1-Propanol
Temperature (°C)					
10	56.3	51.8	44.8	28.4	55.0
20	84.5	72.7	65.2	46.8	80.8
30	100	100	87.0	78.0	99.3
40	90.1	97.7	100	100	100
50	47.2	19.1	58.5	73.4	52.6

Table 6

Effect of Metal Ions on AADH Activity	
Metals (5 mM)	Relative Activity (%)
$CuCl_2$	0
$MnCl_2$	0
$FeCl_3$	2.6
$ZnCl_2$	9.6
$FeCl_2$	11.3
$CoCl_2$	16.5
$NiSO_4$	23.0
$MgCl_2$	93.0
$CaCl_2$	97.8
Control	100.0

Table 7

Effect of Inhibitors on AADH Activity	
Inhibitors (mM)	Relative Activity (%)
EDTA (5)	12.6
EGTA (5)	10.9
NaF (1)	101.3
Quinine (1)	98.7
NEM* (2)	91.7
Hydroxyamine (0.5)	98.3
ICH_2COONa (1)	99.1
Control	100.0

* N-Ethylmaleimide

Table 8

Summary of Purification Steps of AADH				
	Total(a) activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cell free extract(b)	5292	16380	0.32	100
Soluble fraction(c)	4960	12180	0.41	93.5
PEG 6000 supernatant (d)	5333	5501	0.97	100.8
DEAE-Toyopearl (e)	4120	832	4.95	77.9
Q-Sepharose (f)	4468	664	6.73	84.4

(a) The "activity" was measured by the method described in 1) under Enzyme activity on pages 2 and 3.
(b) The "Cell free extract" was prepared by the method described in Example 1 (2) on page 10.
(c) The "Soluble fraction" was prepared by the method described in Example 1 (2) on page 10.
(d) The "PEG 6000 supernatant" was prepared by the method described in Example 1 (3) on page 10.
(e) "DEAE-Toyopearl" was prepared by the method described in Example 1 (4) on pages 10-11.
(f) "Q-Sepharose" was prepared by the method described in Example 1 (5) on page 11.

Table 9

Products from Various Substrates by AADH	
Substrate	Products
Sorbose	Sorbosone 2-KGA
Sorbosone	2-KGA
Sorbitol	Glucose Gluconate Sorbose
Glucose Fructose	Gluconate 2-Keto-D-gluconate

Claims

1. Alcohol/aldehyde dehydrogenase having the following physicochemical properties:
   a) Optimum pH: about 7.0 - 9.0
   b) Optimum temperature: about 20° - 40°C
   c) Molecular weight: 135,000 ± 5,000 dalton
   (Consisting of two subunits in any combination of such α-subunit and β-subunit, each having a molecular weight of 64,500 ± 2,000 and 62,500 ± 2,000, respectively)
   d) Substrate specificity: active on primary and secondary alcohols and aldehydes
   e) Prosthetic group: pyrroloquinoline quinone
   f) Isoelectric point: about 4.4

2. The alcohol/aldehyde dehydrogenase according to claim 1, which is derived from a microorganism belonging to the genus Gluconobacter which is capable of producing the alcohol/aldehyde dehydrogenase having the properties as given in claim 1.

3. The alcohol/aldehyde dehydrogenase according to claim 2, wherein the microorganism is Gluconobacter oxydans having the identifying characteristics of the strain Gluconobacter oxydans DSM No. 4025 (FERM BP-3812).

4. The alcohol/aldehyde dehydrogenase according to claim 3, wherein the microorganism corresponds to Gluconobacter oxydans DSM No. 4025 (FERM BP-3812), a functional equivalent, subculture, mutant or variant thereof.

5. A process for producing an alcohol/aldehyde dehydrogenase having the following physicochemical properties:
   a) Optimum pH: about 7.0 - 9.0
   b) Optimum temperature: about 20° - 40°C
   c) Molecular weight: 135,000 ± 5,000 dalton
   (Consisting of two subunits in any combination of such α-subunit and β-subunit, each having a molecular weight of 64,500 ± 2,000 and 62,500 ± 2,000, respectively)
   d) Substrate specificity: active on primary and secondary alcohols and aldehydes
   e) Prosthetic group: pyrroloquinoline quinone
   f) Isoelectric point: about 4.4

   which comprises cultivating a microorganism belonging to the genus Gluconobacter, which is capable of producing the alcohol/aldehyde dehydrogenase having the above properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganisms, isolating and purifying the enzyme from the cell free extract of the disrupted cells of the microorganism.

6. A process according to claim 5, wherein the microorganism is Gluconobacter oxydans having the identifying characteristics of the strain Gluconobacter oxydans DSM No. 4025 (FERM BP-3812).

7. A process according to claim 6, wherein the microorganism corresponds to Gluconobacter oxydans DSM No. 4025 (FERM BP-3812), a functional equivalent, subculture, mutant or variant thereof.

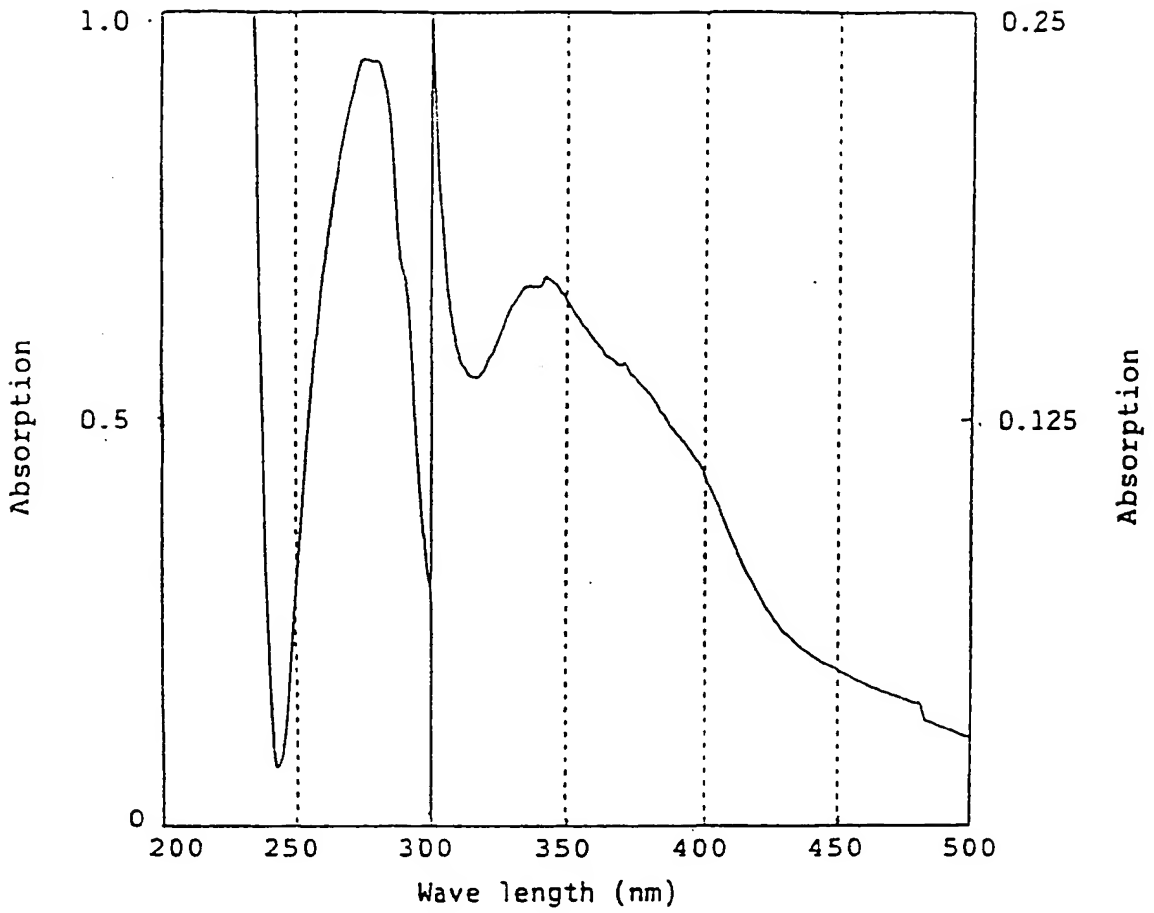
8. A process for producing an aldehyde, a ketone and/or a carboxylic acid from an alcohol or an aldehyde, which comprises contacting the alcohol and/or the aldehyde with
(i) the alcohol/aldehyde dehydrogenase having the following physicochemical properties
a) Optimum pH: about 7.0 - 9.0
b) Optimum temperature: about 20° - 40°C
c) Molecular weight: 135,000 ± 5,000 dalton
(Consisting of two subunits in any combination of the α-subunit and the β-subunit, each having a molecular weight of 64,500 ± 2,000 and 62,500 ± 2,000, respectively)
d) Substrate specificity: active on primary and secondary alcohols and aldehydes
e) Prosthetic group: pyrroloquinoline quinone
f) Isoelectric point: about 4.4
or
(ii) a microorganism belonging to the genus Gluconobacter which is capable of producing the alcohol/aldehyde dehydrogenase in an aqueous nutrient medium under an aerobic condition, or
(iii) a cell free extract of said microorganism
in the presence of an electron acceptor, and isolating the resulting aldehyde, ketone or carboxylic acid from the reaction mixture.

9. A process for producing an aldehyde, a ketone and/or carboxylic acid according to claim 8, wherein the microorganism is Gluconobacter oxydans having the identifying characteristics of the strain Gluconobacter oxydans DSM No. 4025 (FERM BP-3812).

10. A process for producing an aldehyde, a ketone and/or carboxylic acid according to claim 9, wherein the microorganism corresponds to Gluconobacter oxydans DSM No. 4025 (FERM BP-3812) a functional equivalent, subculture, mutant or variant thereof.

11. A process according to claim 8, 9 or 10, wherein said contact is effected by incubating the alcohol in the presence of said microorganism.

Fig. 1. Absorption Spectrum of AADH



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(54) Alcohol/aldehyde dehydrogenase

(57) The present invention relates to a novel alcohol/aldehyde dehydrogenase (hereinafter referred to as AADH), a process for producing the same and a process for producing aldehydes, carboxylic acids and ketones, especially 2-keto-L-gulonic acid (hereinafter referred to as 2-KGA) utilizing said enzyme.

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
Y	EP-A-0 366 922 (F. HOFFMANN-LAROCHE AG) * the whole document * ---	1-11	C12N9/02 C12N9/04 C12P7/60 C12P7/24 //(C12P7/24, C12R1:01)
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	15 June 1995	Gac, G

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A	WO-A-89 06688 (F. HOFFMANN-LAROCHE AG) * abstract * * page 2, line 26 * * page 4 - page 5 * * page 7 - page 8 * * page 33 - page 36 *	1-11	
A	EP-A-0 248 401 (F. HOFFMANN-LAROCHE AG) * the whole document *	1-11	
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